

Phenotypic, molecular, and symbiotic characterization of the rhizobial symbionts isolated from *Acacia saligna* grown in different regions in Morocco: An multivariate approach

Sara LEBRAZI (✉ s.lebrazi@gmail.com)

Sidi Mohamed Ben Abdellah University

Mouhcine FADIL

Sidi Mohamed Ben Abdellah University

Marwa CHRAIBI

Sidi Mohamed Ben Abdellah University

Kawtar FIKRI BENBRAHIM

Sidi Mohamed Ben Abdellah University

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Abstract

The introduced species *Acacia saligna* is a very promiscuous host as it can be efficiently nodulated with a wide range diversity of rhizobia taxa, including both fast and slow-growing strains. Fourteen nitrogen (N)-fixing bacteria were isolated from root nodules of wild *Acacia saligna* growing in distinct geographic locations in Morocco and were examined for their symbiotic efficiency and phenotypic properties. Multivariate tools, such as principal component analysis (PCA) and hierarchical clustering analysis (HCA), were used to study the correlation between phenotypic and symbiotic variables and discriminate and describe the similarities between different isolated bacteria with respect to all the phenotypic and symbiotic variables. Phenotypic characterization showed a variable response to extreme temperature, salinity and soil pH. At the plant level, the nodulation, nitrogen fixation, and the shoot and root dry weights were considered. The obtained results show that some of the tested isolates exhibit remarkable tolerances to the studied abiotic stresses while showing significant N₂ fixation, indicating their usefulness as effective candidates for the inoculation of acacia trees. The PCA also allowed showing the isolates groups that present a similarity with evaluated phenotypic and symbiotic parameters. The genotypic identification of N₂-fixing bacteria, carried out by the 16S rDNA approach, showed a variable genetic diversity among the 14 identified isolates, and their belonging to three different genera, namely *Agrobacterium*, *Phyllobacterium* and *Rhizobium*.

Introduction

The massive use of synthetic fertilizers in agriculture is nowadays subject to debates related to both environmental and public health concerns. The addition of such chemicals has induced land desertification and salinization, which reduced productivity and availability of biological resources. Desertification has become an alarming menace in many parts of the world because it leads to an unrecoverable deterioration of the vegetation cover with a consequent decrease in soil fertility (Arora 2018; Lebrazi and Fikri-Benbrahim 2022). In addition to affecting native plant communities, soil degradation also disturbs the symbioses in the plant microsphere, which play a crucial role in fostering plant growth in degraded ecosystems (Requena et al. 2001). Microorganisms associated with crops play an important role in the productivity and sustainability of several natural ecosystems and agro-ecosystems. Indeed, rhizobia are soil bacteria group from different genus that are mainly associated with plants of the legume family. These rhizobacteria can induce the development of root nodules and provide their host plants with a significant N amount through their ability to fix atmospheric dinitrogen.

The primordial role of trees in soil protection, litter production, and more globally in the improvement of edaphoclimatic conditions has been widely demonstrated (Ribeiro-Barros et al. 2018). Therefore, the performance of rhizobia for symbiotic nitrogen fixation plays a key role in the selection of field applications. Bacterial strain selection technology aims to improve more efficient strains to replace native soil strains. This provides an effective biotechnological tool for restoring degraded or desert ecosystems. Nodulation and symbiotic nitrogen fixation are highly correlated and related to both plant and symbiotic bacterial genotype. The introduction of tree legumes into cropping systems often involves the selection of

competitive and persistent symbiotic bacteria as inoculants to improve nitrogen fixation and crop yield (Lebrazi and Fikri-Benbrahim 2022). Acacia tree (*Acacia saligna*) is among the most widespread trees in the arid and Saharan regions of southern and south-eastern Morocco due to their ability to adapt to a long dry season (9 to 10 months) (Noumi, 2010) and to the harsh ecological conditions of these areas. The ability of Acacia trees to perform symbiosis with rhizobia that fix atmospheric dinitrogen allows them to improve and maintain soil fertility levels (Bakhoum et al. 2018). By establishing bacterial and mycorrhizal symbioses, woody species have the necessary and sufficient adaptations to grow on very poor and mineral-depleted soils.

Therefore, it is possible to improve the composition of the rhizobial and mycorrhizal communities associated with the trees in order to increase the biomass production by these plants and also contribute to the mitigation of erosion.

The incorporation of Acacia species into reforestation programs based on their high resistance to various biotic and abiotic stress factors, can offer a sustainable reforestation solution in arid and semi-arid areas. In Morocco, the Acacia woodlands cover an area of 1,128,000 ha, including *Acacia mearnsii* in the Mâamora region (Atlantic plain), *A. gummifera* and *Acacia* spp. in the Atlantic and Eastern Meseta (Middle Atlas), *A. gummifera*, *A. raddiana*, *A. seyal* and *A. albida* in the presahara (Souss region) as well as *A. raddiana* and *A. seyal* in the Sahara (Fikri Benbrahim et al. 2014). The increased attention to the utilization of rhizobium as biofertilizers in agricultural systems has allowed the identification of a high number of rhizobacterial strains. To effectively exploit symbiotic N₂ fixation to improve plant production, it is necessary not only to select the best host cultivar, but also to properly and sufficiently characterize the native rhizobia population. On a practical level, the selection of a suitable host plant and complementary microsymbionts depends on the edaphic environment, which is subject to several irregularities depending on the intensity and nature of crops, the geographical environment and the soil conditions. Thus, salinity, pH and temperature perform the most important environmental constraints in plant-rhizobia symbiosis. Therefore, it is necessary to evaluate the environmental factors in order to ensure that the selected symbiotic couple has the necessary aptitudes to establish itself in a given type of soil and meet the defined requirements. Studying the diversity of rhizobia strains nodulating acacia trees and selecting efficient bacteria helps to identify the most appropriate combinations of plants and microsymbionts for use in reforestation programs. Therefore, the exploitation of acacias rhizosphere to promote the restoration of vegetation cover in arid and semi-arid areas seem to be interesting due to their participation in the improvement of soil stabilization and fertility through N₂ transfer to the associated crops.

Thus, the aim of this work was to study the phenotypic and genotypic parameters of rhizobia isolated from root nodules of acacia trees from different sites in Morocco with the aim of selecting efficient strains able to tolerate the different environmental variations while providing a successful N₂ fixation. Multivariate tools (principal component and hierarchical clustering analyses) were used to show correlations between phenotypic and symbiotic variables and describe the similarities between the isolates' origin sites.

Material And Methods

Rhizobial strains' isolation

Root nodules of *Acacia saligna* were collected from eight Moroccan geographic sites having different climates (Table 1). Bacteria were isolated according to the method recommended by Vincent, (1970) and Beck et al., (1993). A digging of about 15–25 cm was conducted around the plant to a minimum depth of 20 to 50 cm to extract part of the root system (Lebrazi et al. 2018).

Table 1
Geographic origin, geodesic coordinates, and climate type of the sampling sites

Isolates	Geographic origin	Latitude longitude altitude	Climate type ^a	Average temperature (°C) ^a	Average rainfall (mm) ^a
I ₁ ; I ₃ ; I ₂₈ ; I ₇₂	Oujda	34°41'12" N 1° 54' 41" W 450 m	BSk (Cold semi-arid climate)	16.7	338
I ₅	Ras El-ma (Nador)	35° 08'10"N 2° 25'30"W 1 m	CSa (Mediterranean climate)	18.2	375
I ₆	Saidia	35° 05' 15" N 2° 17' 16" W 20 m	BSk (Cold semi-arid climate)	17.7	371
I ₁₆	Berkane	30° 25' 12" N 9° 33' 53" W 16 m	BSk (Cold semi-arid climate)	16.7	371
I ₃₉ ; I ₇₀	Fez	34° 02' 13" N 4° 59' 59" W 104 m	CSa (Mediterranean climate)	18	536
I ₅₇	Rabat	34°01' 31" N 6°50'10" W 79m	CSa (Mediterranean climate)	18.1	442

^a Climate type of each region according to the Köppen climate classification system

Isolates	Geographic origin	Latitude longitude altitude	Climate type ^a	Average temperature (°C) ^a	Average rainfall (mm) ^a
I ₇₄	Casablanca	33° 35' 17" N 7° 36' 40" W 27 m	CSa (Mediterranean climate)	17.7	412
I ₇₅ ; I ₇₆ ; I ₇₈	Er-Rachidia	31°55'53" N 4°25'35" W 1039 m	BWh (Desert climate)	19.5	127
^a Climate type of each region according to the Köppen climate classification system					

Then, nodules surface was sterilized with 95% (v/v) ethanol for 30 s, and immersed in mercury chloride (HgCl₂) solution 0.1% (w/v) for 4 min. A succession of three rinses for 10, 15 and 20 min, respectively, was carried out aseptically with sterile distilled water. Surface sterilized nodules were crushed with a few drops of 9‰ NaCl (w/v) under aseptic conditions (Beck et al. 1993). One hundred microliters of the suspension obtained was spread on Petri dishes containing YMA (Yeast, Mannitol Agar) culture medium (Vincent 1970) supplemented with 0.0025% (w/v) of Congo red. The single colonies were selected and restreaked in order to purify them (Jordan 1984). Pure cultures were stored in 20% glycerol at -80°C until subsequent use. (Elbanna et al. 2009; Berrada et al. 2012).

Morphology of the colonies

The colony morphology of the different isolates was verified on the YMA agar medium. After incubation for 3 to 7 days at 28°C, individual colonies were characterized for selected morphological parameters such as size, color, mucosity, transparency, shape, elevation, border and the production capacity of exopolysaccharide gum (Vincent 1970; Berrada et al. 2012).

The rhizobial isolates growth was evaluated on PGA (peptone-glucose-agar) broth medium according to Somasegaran and Hoben, (1994) as a first level of selection due to weak rhizobia grow on this medium. Colonies resulting from pure cultures of the different isolates were subjected to microscopic observation after a Gram staining to allow more specific identification of these isolates and their bacterial walls to classify them into Gram positive or negative bacteria groups.

The strains' ability to alkalinize or acidify the YMA medium was evaluated by adding 0.0025% (w/v) bromothymol blue as color indicator. After incubating the inoculated plates at 28°C for 24 hours, the color change of the medium was observed. A yellow coloration indicates an acidic reaction and the rhizobia

are fast growers. Dark blue coloration indicates a basic reaction and slow rhizobia growers. The isolates were divided into different morphotypes groups based on the observed morpho-cultural characteristics.

Symbiotic efficiency of bacterial isolates

The nodulation ability of the studied isolates was examined by inoculating *A. saligna* seedlings grown on pots containing sterile sandy-loam soil with three seeds per pot. Seeds were first surface sterilized by rinsing in 95% (v/v) ethanol, immersing for 4 min in 0.2% HgCl₂ (w/v), and washing three times in sterile distilled water. They were further scarified with 95% H₂SO₄ and germinated on 0.9% agar in obscurity (Fikri-Benbrahim et al. 2017). One week after germination, seedlings were inoculated with approximately 1 ml of a freshly prepared bacterial suspension (10⁸ UFC/ml) of each isolate. The plants were fed three times a week alternately with a sterilized N-free nutrient solution (Broughton and Dilworth 1971) and sterile distilled water. Non-inoculated plants were considered as N-free control (C₀). Nitrogen control (N_C), receiving weekly 0.5% KNO₃ (w/v) as N source, was considered a non-inoculated control. Each treatment was prepared in triplicate. Six months after inoculation, plants were harvested to determine the infectivity and effectivity of isolates. Root nodules were recorded on each individual plant. The plant's shoot and root dry weights were assessed. The average dry weight of plants inoculated with the same strain was used to estimate the relative efficiency (RE), which is expressed as the percentage of shoot dry weight of inoculated plants compared to the dry weight of nitrogen control (El Hilali et al. 2007). The Kjeldahl method was then used to measure the nitrogen content of the aerial part (Nelson and Sommers 1973).

Evaluation of rhizobial tolerance to abiotic stresses

Rhizobia isolates were evaluated for their ability to grow under different environmental stresses (pH, salinity and temperature) conditions in YMA culture medium. For pH tolerance of rhizobia isolates, 10 µl of bacterial suspension of each rhizobia isolates (10⁸ CFU/ml) were seeding on YMA culture medium adjusted at different pH values (4.8, 5.8, 6.8, 8.8, and 10.0). The plates were incubated at 28°C for one week (Küçük et al. 2006). Salinity tolerance was evaluated in YMA culture medium with increased concentrations of sodium chloride (NaCl) ranging from 855 to 1710 mM (Lebrazi et al. 2018). Finally, the tolerance of rhizobial isolates to different temperatures was analyzed in YMA plates (pH 7.0) incubated at 6, 14, 28, 37, 44 and 54°C (Hung et al. 2005). All tests were carried out in triplicate.

Amplification and sequencing of 16S rRNA gene

Genomic DNA extraction is directly performed from bacterial colonies by thermal shock technique. Universal primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1540 R (5'-AAG GAG GTG ATC CAG CC-3') were used for polymerase chain reaction (PCR) amplification of a 1500-bp segment of the 16S rRNA gene (Edwards et al. 1989; Lebrazi et al. 2020). Each 50 µL reaction comprised 1.0 µL of the cell lysate (approximately 20ng DNA), 1.25 U of GoTaqR G2 DNA polymerase (Promega), 1X reaction buffer, 5%

Dimethyl sulfoxide (DMSO), 0.2 mM dNTPs as well as 0.15 μ M of each primer. The PCR protocol was conducted as follows: an initial cycle of denaturation at 95°C (4 min), 35 cycles of denaturation at 95°C (1 min each), annealing at 55°C (1 min), extension at 72°C (2 min), and the last extension step fixed in 7 min. Sanger sequencing was used to determine the nucleotide sequence for both strands of PCR products. DNA sequences were compared to the GenBank database by basic local alignment search tool (BLAST) requests using the blast-n algorithm and the highly similar sequence optimization (megablast) (Lebrazi et al. 2018).

Multivariate statistical analyses

In order to explore the possible relationships between the plant environmental stressors and symbiont characteristics, we perform the principal component analysis (PCA) to obtain a projection of variables on the factorial plane.

The considered phenotypic variables were temperature, NaCl, and pH tolerance. At the plant level, the variables of nodulation, aerial parts and root growth, as well as the nitrogen content of the aerial parts of the plant were taken into account.

The phenotypic variables considered in PCA were pH tolerance, salinity, and temperature, while, at the plant level, nodulation, shoot and root growth, as well as N content were taken into account to this analysis. The hierarchical cluster analysis (HCA) was used to better visualize the clustering of locations. Pearson's correlation test was used to evaluate the correlation between studied variables. All statistical tests were carried out using JMP Software V 14 (Bridges 1966).

Results

Morpho-cultural characteristics of isolates

The studied isolates were identified as Gram-negative bacilli, which were unable to absorb congo red present on YMA medium and grow on PGA medium. The isolates' colonies presented the same morphology on the YMA medium: circular, convex, smooth, translucent, creamy to transparent texture. All isolates were considered fast acid producers as they changed the bromothymol blue indicator from deep green to yellow in the YMA-BTB. Moreover, 90% of the studied isolates presented a mucoid texture indicating the production of exopolysaccharides.

Evaluation of symbiotic parameters of isolates

Isolates' efficiency was determined by examining the presence of red coloration in the nodules. Great variability in the infectivity of isolates was detected (Table 2). Even the plants receiving the same inoculum concentrations from different isolates, the number of nodules per plant was different from one plant to another. I₁ was the most infective isolate and induced 81 nodules per plant, while the least

infective one (I₅₇) caused the development of only 20 nodules per plant. Some plant-rhizobia partners were superior to nitrogen control in terms of plant dry weight, with highlights for plants inoculated with I₁ and I₇₈ (Table 2). Plants inoculated with I₁ exhibited a shoot dry weight of 15.32 g plant⁻¹ and a relative efficiency of 127.49%, indicating that this isolate is the most efficient among others. The least efficient isolate was I₅ with relative efficiency of the 45.25%. The relative efficiency of non-inoculated control was 10.52%. In general, the isolates I₁, I₂₈, I₃₉ and I₇₈ were very efficient showing a higher relative efficiency (> 101%). The total N content showed significant differences between the tested isolates (p-value < 0.0001) (Table 2). Plants inoculated with I₁, I₃₉, I₇₀, and I₇₈ accumulated more nitrogen and present 4.90%, 4.49%, 4.43%, and 4.37% of N content, respectively. On the other hand, plants inoculated with I₅₇ displays the lowest N content (1.42%).

Table 2

Plant growth parameters: (SDW: Shoot dry weight, RDW: root dry weight, nodule number (Nod N), and RE: Relative efficiency) values of *A. cyanophylla* evaluated six months after plants inoculation.

Isolate	SDW (g.plant ⁻¹)	RDW (g.plant ⁻¹)	RE	Nod N.plant ⁻¹	N (%)
I ₁	15,32 ± 0,55	9,97 ± 0.74	127,49 ± 4,64	81 ± 9.19	4,9 ± 0.16
I ₃	7,25 ± 1.15	4,84 ± 1.71	66,97 ± 9;64	37 ± 3.53	2,33 ± 0.08
I ₅	5,44 ± 0,77	3,12 ± 0.14	45,25 ± 6,47	57 ± 3.53	2,77 ± 0.12
I ₆	8,72 ± 0,33	6,01 ± 1.22	72,54 ± 2,82	56 ± 5.65	3,82 ± 0.12
I ₁₆	5,54 ± 1,15	2,89 ± 0.20	46,13 ± 9,58	56 ± 5.65	3,44 ± 0.41
I ₂₈	13,25 ± 0,55	8,75 ± 0.28	110,27 ± 4,64	67 ± 4.24	2,97 ± 0.16
I ₃₉	12,22 ± 2,4	7,38 ± 0,09	101,66 ± 9,94	75 ± 12	4,49 ± 0.24
I ₅₇	5,96 ± 2,46	3,82 ± 0,56	49,58 ± 2,23	20 ± 2,82	1.42 ± 0.12
I ₇₀	9,74 ± 10,58	8,28 ± 2,63	81,03 ± 4,82	70 ± 2,82	4,43 ± 0.08
I ₇₂	9,41 ± 1,19	8,15 ± 0,60	78,32 ± 4,94	80 ± 12,02	3,7 ± 0.12
I ₇₄	6,96 ± 1,35	5,05 ± 4,06	57,94 ± 11,23	70 ± 11,31	3,67 ± 0.08
I ₇₅	9.83 ± 0,55	6,49 ± 4,17	81,82 ± 4,64	54 ± 3,53	2.91 ± 0.5
I ₇₆	11,16 ± 2,35	8,38 ± 6,28	92,84 ± 19,53	56 ± 5,65	3,76 ± 0.04
I ₇₈	14,19 ± 2,07	10,78 ± 2,75	118,09 ± 17,23	89 ± 7,77	4,37 ± 0.09
T _N	12.02 ± 1.13	5.82 ± 0.24	100,04 ± 9,47	0.00 ± 0.00	-
T ₀	1.26 ± 0.06	0.37 ± 0.14	10,52 ± 0,52	0.00 ± 0.00	-
P-value	0.0000	0.0000	0.0000	0.0000	0.0000
T ₀ = non-nitrogenated control, T _N = Control fertilized with KNO ₃					

Tolerances of bacterial isolates to pH, salinity and temperature

Rhizobia isolates were able to tolerate higher salinity level (Table 3), however the maximum salinity tolerance varied greatly depending on the isolate. The ability to survive at temperatures between 14 and 28°C was observed in all tested isolates, while a successful growth within the range of 6 to 44°C has been recorded for six isolates (I₁, I₃, I₅, I₇₂, I₇₄, and I₇₆). Finally, the growth evaluation at different pH values showed optimum growth of all isolates at neural and slightly acidic pH (4.8 to 6.8). I₁, I₃₉, I₇₀, I₇₄, and I₇₆ were able to grow in a broad pH range (4.8 to 10.0) (Table 3). I₁ and I₇₆ showed the combined ability to grow well under all evaluated stress conditions.

Table 3
Tolerance of the tested isolates to different abiotic factors (salinity, pH, temperature).

Isolate	Maximum temperature (°C)	Maximum NaCl concentration (mM)	pH
I ₁	6; 44	1710	4.8; 10
I ₃	6; 44	1710	4.8; 8.8
I ₅	6; 44	855	5.8; 6.8
I ₆	6; 28	1710	5.8; 6.8
I ₁₆	6; 28	1710	5.8; 8.8
I ₂₈	6; 28	1710	5.8; 6.8
I ₃₉	6; 37	1710	4.8; 10
I ₅₇	14; 28	855	5.8; 8.8
I ₇₀	14; 28	855	4.8; 10
I ₇₂	6; 44	1710	4.8; 8.8
I ₇₄	6; 37	1196	4.8; 10
I ₇₅	14; 28	1710	5.8; 8.8
I ₇₆	6; 44	1710	4.8; 10
I ₇₈	6; 44	1196	5.8; 8.8

PCA and HCA

The PCA explain 72% of all data variability and two principal components (PC), being PC1 and PC2 explained 53.1% and 19.3%, respectively, of all data variation. A positive correlation between the four symbiotic parameters (SDW, RDW, nodulation and N content) were well explained by the first component (PC1). These results were confirmed by the Pearson correlation test showed in Table 4. The scores plotted

below (Fig. 2) displays the provenances of samples that present a similarity according to the studied parameters. Thus, there was possible detect two large groups of samples. The group A was formed by Er-rachidia 2, Er-rachidia 3, Fez 1, Fez 2, Oujda 1, Oujda 3 and Oujda 4, while the group B consists from the following provenances: Berkane, Casablanca, Errachidia 1, Nador, Oujda 2 and Saidia. Hierarchical clustering analysis (HCA) was used to make an advanced classification of the isolates' different origins (Fig. 3). The two large classes detected by PCA analysis (groups A and B) were subdivided into five subclasses by HCA, as follow (Fig. 3): (subclass 1) Er-Rachidia 2, Er-Rachidia 3, Fez 1, Oujda 1 and Oujda 4; (subclass 2) Fez 2 and Oujda 3; (subclass 3) Berkane, Er-Rachidia 1, Oujda 2 and Saidia; (subclass 4) Casablanca and Nador; and (subclass 5) Rabat.

Table 4
Observed correlations between the different phenotypic and symbiotic parameters

	SDW mg.plant⁻¹	RDW mg.plant⁻¹	Nod N.plant⁻¹	N(%)	NaCl	pH	Temperature
SDW mg.plant ⁻¹	1						
RDW mg.plant ⁻¹	0,9369*	1					
Nod N.plant ⁻¹	0,6496*	0,7081*	1				
N(%)	0,6337*	0,6665*	0,8653*	1			
NaCl	0,381	0,2433	0,1757	0,2331	1		
pH	-0,4408	-0,4462	-0,2626	-0,043	0,0465	1	
Temperature	0,3475	0,4059	0,5777*	0,4532	-0,0374	0,2205	1
* : Statistically significant correlation							

Using the biplot of scores and loadings (Fig. 4), the relationships between the individual clusters and the symbiotic parameters was detected. Thus, it was observed that rhizobia belonging to the large group A induced high values of the inoculated plants' parameters (SDW, RDW, nodule number, N content) and possess high salinity and temperature tolerance. At the same time, those corresponding to group B are characterized by lower values of the plant's parameters and tolerance to high pH levels.

Sequencing of 16S rRNA gene

The 16S rDNA analysis showed that the sequenced isolates belonged to the genera *Agrobacterium*, *Phyllobacterium* and *Rhizobium*. BLAST homology searches indicated precise matches with the strain

sequences present in GenBank (> 99%) for both sequences resulting from the forward and reverse sequencing reactions.

Discussion

Abiotic stress is a common occurrence in the legume-rhizobium symbiosis, which significantly affects the nodulation process therefore nitrogen fixation (Basu and Kumar 2020).

Tolerance to abiotic stresses makes rhizobia very valuable inoculums to legumes grown in arid and semi-arid zones. Overall, our study revealed that the examined strains were quite tolerant to salinity in vitro, as some strains tolerated concentrations up to 1710 mM NaCl (corresponding to 10% NaCl). These results corroborated previous researches in which rhizobia were isolated from woody legumes in general and dryland acacia tree species in specific could tolerate such as higher salinity (Zahran 1999; Mohamed et al. 2000; Essendoubi et al. 2007; Diouf et al. 2007; Boukhatem et al. 2012). The range of salinity tolerance in rhizobia can be considerably different among species and even among strains of the same species (Missbah El Idrissi et al. 2021). Zerhari et al. (2000) reported that fast-growing rhizobia isolated from some *Acacia* species were tolerant to high NaCl concentrations compared to slow-growing strains. However, Assefa, (1993) found that some strains (slow-growing *Rhizobium*) from woody legumes were more tolerant to NaCl than fast-growing species. Other researches have suggested that growth rate and salt tolerance are not (Fikri-Benbrahim et al. 2017)(Fikri-Benbrahim et al., 2017) correlated with growth rate (Zerhari et al. 2000) but are for physiological and biochemical mechanisms (Botsford and Lewis 1990; Gouffi et al. 1999).

The isolated strains demonstrated relatively excellent temperature tolerance, with six strains tolerating temperatures between 6 and 44°C, which is concordant with previous studies showing that rhizobia were able to grow within a broad temperature range (Maâtallah et al. 2002; Fikri-Benbrahim et al. 2017). Rhizobia are considered mesophilic, with optimal growth temperatures of 28 to 30°C (Sharma et al. 2017). The maximum temperatures (T_{max}) for growth of free-living rhizobia in soil are between 35 and 45°C (Zhang et al. 1991; Zahran et al. 2012). Zerhari et al., (2000) and Assefa, (1993) also showed the ability of some rhizobia strains isolated from woody legumes to tolerate temperatures ranging from 4 to 43°C. But, even though rhizobia grow at elevated temperatures, this does not indicate that they are efficient N₂ fixers (Fentahun et al. 2013; Lebrazi et al. 2018).

The results of previous research demonstrating rhizobia's ability to grow in a diverse range of soil pH are corroborated by the evaluated strains' tolerance to different pH values ranging from (4.0–10.0), with optimal growth of all tested isolates at neutral and slightly acidic pH (4.8–6.8) (Küçük and Kıvanç 2010; Youseif et al. 2014; Lebrazi et al. 2018) even at pH 12.0 (Surange et al. 1997). Rhizobia strains contain variants that can be useful in tolerating abiotic stresses such as temperature extremes, pH and salinity. The exploration of tolerant rhizobium strains is predicted to increase plant growth and yield, even under a combined stresses condition.

The nodules' diagnosis highlights the presence of local native rhizobia able to nodulate *Acacia*, for all the fourteen samples. A variability of *Acacia*'s nodulation and growth (shoot and root) is to be noted on all the studied bacteria. Specific variability in the efficiency of rhizobial symbiosis translates into variability in effectiveness (aerial biomass) and infectivity (nodules biomass). The criteria generally considered as determining in the selection of symbiotic bacteria concern the relationship of bacteria with plant (compatibility) or with the soil (adaptation). Strain compatibility with the host plant is defined according to infectivity and effectivity, which vary to the bacterial and plant genotype. Some rhizobial strains can be infectious and not effective but only the strains that are both infectious and effective are considered to be compatible. Using a multivariate statistical PC analysis, we were able to simultaneously evaluate the seven variables considered by condensing them into two principal components with minimum mathematical information loss. The principal components are used as axes where the data can be plotted and visualized structurally. An efficient rhizobial symbiosis is recognized by an overall statistically significant correlation between plant growth and nodulation. Significant correlations were found between dry weight of the *Acacia* aerial part and the number of nodules obtained by each rhizobial isolate (Fig. 1), indicating that the increased biomass recorded in plants inoculated with isolates was due to its increased nodulation potential, and not inevitably only to a greater N-fixing capacity (strain-specific). These findings proved that plants inoculated with the studied isolates (I₁, I₂₈, I₃₉ and I₇₈) had greater aerial biomass than the N-fertilized control plants and that these four strains resulted in an increase in root biomass compared to N-fertilized plants. This effect on root growth by inoculated rhizobia could promote plant tolerance to nutritional and drought stress through the enhancement of nutrient and water uptake by producing a more extensive root system. The spatial variability of nodulation and growth observed in the different tested strains proves that it is difficult to build a model or draw conclusions by observing nodulation parameters only on a single plot. Each plot has its own specificities that influence legume nodulation. Soil, physicochemical proprieties, geomorphy, previous crops, environment, climate, plant and microorganisms are all edapho-climatic, microbiological and physiological factors that influence the interactions of legumes with the soil.

PCA was used to explore the variation in the distribution of isolates in the PC1-PC2 space according to the different studied stress tolerance phenotypes and symbiotic parameters as well as their geographic sites of origin. The phenotypic and symbiotic characteristics of strains from the same site varied considerably. Thus, as clearly demonstrated by the PCA (Fig. 4), the absence of an effect of the corresponding sampling sites on the different strains was highlighted, as no similarity could be found between the results of strains of the same geographical origin. Infact, the rhizobial community at a sample site may be more heterogeneous, and the rhizosphere of one plant may have the same rhizobial diversity as the entire sample region, hosting many rhizobial strains (Moschetti et al. 2005). Nevertheless, it was possible to detect those strains of the different geographical origins showed similar responses, as is the case for the Oujda1 and Errachidia3 strains. In the same way, the study of Boukhatem et al. (2012) on the diversity of rhizobia associated with *Acacia* in arid and semi-arid regions of Algeria showed the absence of correlation between the in vitro tolerances of the strains to different abiotic stress factors and their origin's areas.

Concerning the molecular characterization, 16S rRNA gene sequencing has been widely employed to investigate rhizobial diversity associated with legume species (Cardinale et al. 2008; Muindi et al. 2017; Lebrazi et al. 2018), especially *A. saligna* (Zerhari et al. 2000; Amrani et al. 2010). The sequencing result indicates that the studied isolates were identified as members of *Agrobacterium*, *Phyllobacterium* and *Rhizobium* genera (Table 5). Earlier studies have reported that strains associated with *Acacia* spp. in Africa, can belong to *Agrobacterium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (De Lajudie et al. 1994; Odee et al. 2002; Amrani et al. 2010).

Table 5
Sequence analysis of 16S rDNA of the studied isolates

Isolate	Accession (no)	Homology to the Identity reference strains	Identity (%)
I ₁	MF767517.1	<i>Rhizobium pusense</i>	99
I ₃	MF173082.1	<i>Rhizobium</i> sp.	99
I ₅	EF638791.1	<i>Rhizobium</i> sp.	99
I ₆	EF437255.1	<i>Rhizobium</i> sp.	99
I ₁₆	KJ748400.1	<i>Rhizobium</i> sp.	99
I ₂₈	LC133652.1	<i>Phyllobacterium</i> sp.	99
I ₃₉	KX884928.2	<i>Rhizobium</i> sp.	99
I ₅₇	KF312292.1	<i>Phyllobacterium myrsinacearum</i>	99
I ₇₀	DQ303313.1	<i>Rhizobium</i> sp.	99
I ₇₂	MH669301.1	<i>Rhizobium</i> sp.	99
I ₇₄	MT573182.1	<i>Rhizobium</i> sp.	99
I ₇₅	CP019702.1	<i>Agrobacterium rhizogenes</i>	99
I ₇₆	AB921279.1	<i>Agrobacterium</i> sp.	99
I ₇₈	JX514418.1	<i>Rhizobium</i> sp.	99

Recently, nitrogen-fixing bacteria have also been described in other genera of alphaproteobacteria, including *Phyllobacterium* (Zakhia et al. 2006; De Meyer et al. 2015). This finding suggests that the genes responsible for symbiosis with legumes are horizontally transmissible and function across a relatively wide range of bacterial taxa. According to previous research, several *Acacia* species have been nodulated by the genus *Phyllobacterium*. (Hoque et al. 2011; Crisóstomo et al. 2013). In some known exceptional cases, 16S rDNA sequencing cannot differentiate between some genera, as with *Agrobacterium* species

and *Rhizobium* species. Although *Rhizobium* is the nitrogen-fixing symbiont genus and *Agrobacterium* is the plant pathogen genus, these two genera are phylogenetically intertwined with each other and their 16S rDNA sequences cannot separate them. Young et al. (2003) proposed that all *Agrobacterium* species could more correctly be considered members of *Rhizobium*. However, it was noticed that the reclassification of *Agrobacterium* into *Rhizobium* might be considered premature, excluding the ancient *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) that clearly is a member of the genus *Rhizobium* (Farrand et al. 2003; Velázquez et al. 2007; Ramírez-Bahena et al. 2014). *Agrobacterium* strains have been isolated from root nodules of beans and other legumes (De Lajudie et al. 1999; Hameed et al. 2003; Mrabet et al. 2006; Wang et al. 2006). Unfortunately, on further examination of nodulation, these isolates were unable to nodulate their original host but otherwise they are able to colonize pre-formed nodules. The mechanism whereby these isolates are incorporated into the nodules is currently not well understood. It appears that genes other than those implicated in the symbiotic process should probably be involved. Transient acquisition of a symbiotic plasmid was thought to be an appropriate explanation. De Lajudie et al. (1999) showed that these *Agrobacterium* isolates are non-pathogenic. Mrabet et al. (2006) tested the effect of inoculation of *Phaseolus vulgaris* with *Agrobacterium* isolates on nodulation rate and vegetative yield; and showed that inoculation with these isolates negatively affected nodulation and plant growth. A similar study was conducted, under semi-natural conditions, by Chihaoui et al. (2015) using *Agrobacterium* sp. 10C2 a non-symbiotic and non-pathogenic nodule-endophyte strain isolated from root nodules of *P. vulgaris*. Inoculation with strain 10C2 increased the nodule number and plant biomass. *Agrobacterium rhizogenes* is a plant's pathogen which is able to promote hairy roots in a wide range of plants by integrating the T-DNA root inducing plasmid (P_{RI}) into the plant genome (Gutierrez-Valdes et al. 2020). This results in massive production of adventitious roots. The hairy roots can ensure a continuous source of secondary metabolites which are therefore widely preferred for the production of various chemicals for cosmetics, pharmaceuticals and food additives (Chandran and Potty 2008).

Therefore, according to the results obtained during this study, it can be assumed that the selected isolates could be beneficial for their use as potential agents and promoters of plant growth and development. However, the good results obtained in vitro cannot always be reliably reproduced under field conditions. Hence, further field trials using these bacteria would be necessary to understand their potential in the agro-ecosystem as PGPRs.

Conclusion

The use of tree legumes, such as acacias, to promote canopy restoration in arid and semi-arid areas appears particularly interesting due to their potential to adapt to a long dry season and harsh ecological conditions. This work, mainly based on studying the diversity of rhizobia isolated from root nodules of *A. saligna* from different regions of Morocco, allowed us to select efficient rhizobia capable of fixing nitrogen and tolerating various environmental constraints. PCA was used to discriminate and describe the similarities between the different origin sites with respect to all measured phenotypic and symbiotic variables for the studied isolates. The strains' tolerance to these factors needs to be further evaluated in symbiosis with the host plant. In this perspective, further complementary investigations on tree species

provenances would be interesting to further explain the responses of this symbiosis to particular edaphoclimatic conditions.

Abbreviations

CFU Colony Forming Unit

HCA Hierarchical clustering analysis

HgCl₂ Mercury chloride

NaCl Sodium chloride

PCA Principal component analysis

KNO₃ Potassium nitrate

PCR Polymerase chain reaction

PGA Peptone-glucose-agar

RDW Root dry weight

RE Relative efficiency

SDW Shoot dry weight

YMA Yeast, Mannitol Agar

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Figures

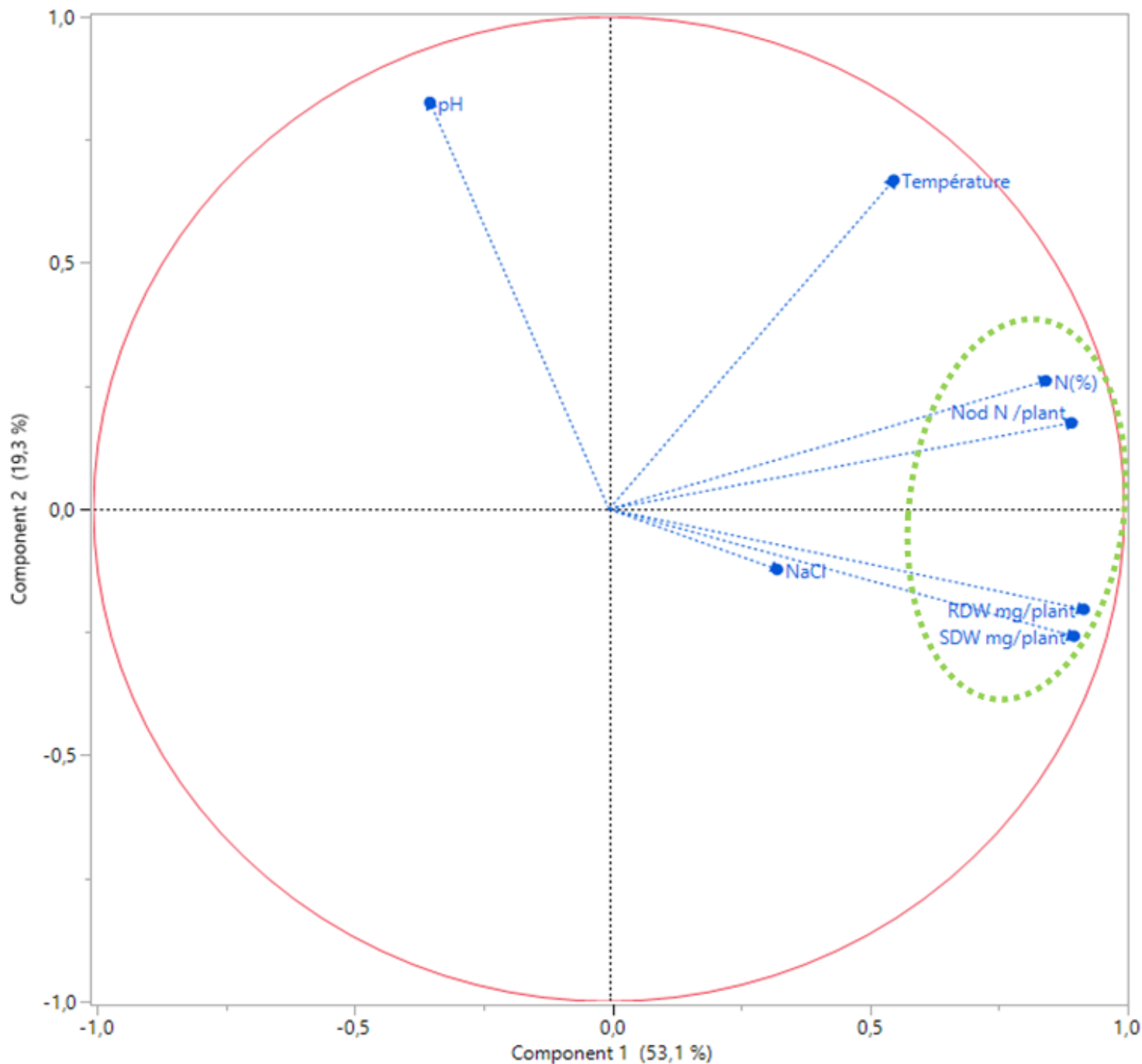


Figure 1

Graph of correlations between phenotypic and symbiotic parameters according to the first two components.

Vector correlation plot between the examined variables, i.e. stress tolerance (NaCl, pH and temperature tolerance) and symbiotic parameters: shoot dry weight (mg plant^{-1}); root dry weight (mg plant^{-1}); number of nodules (N Nod plant^{-1}) and percentage of fixed nitrogen N (%).

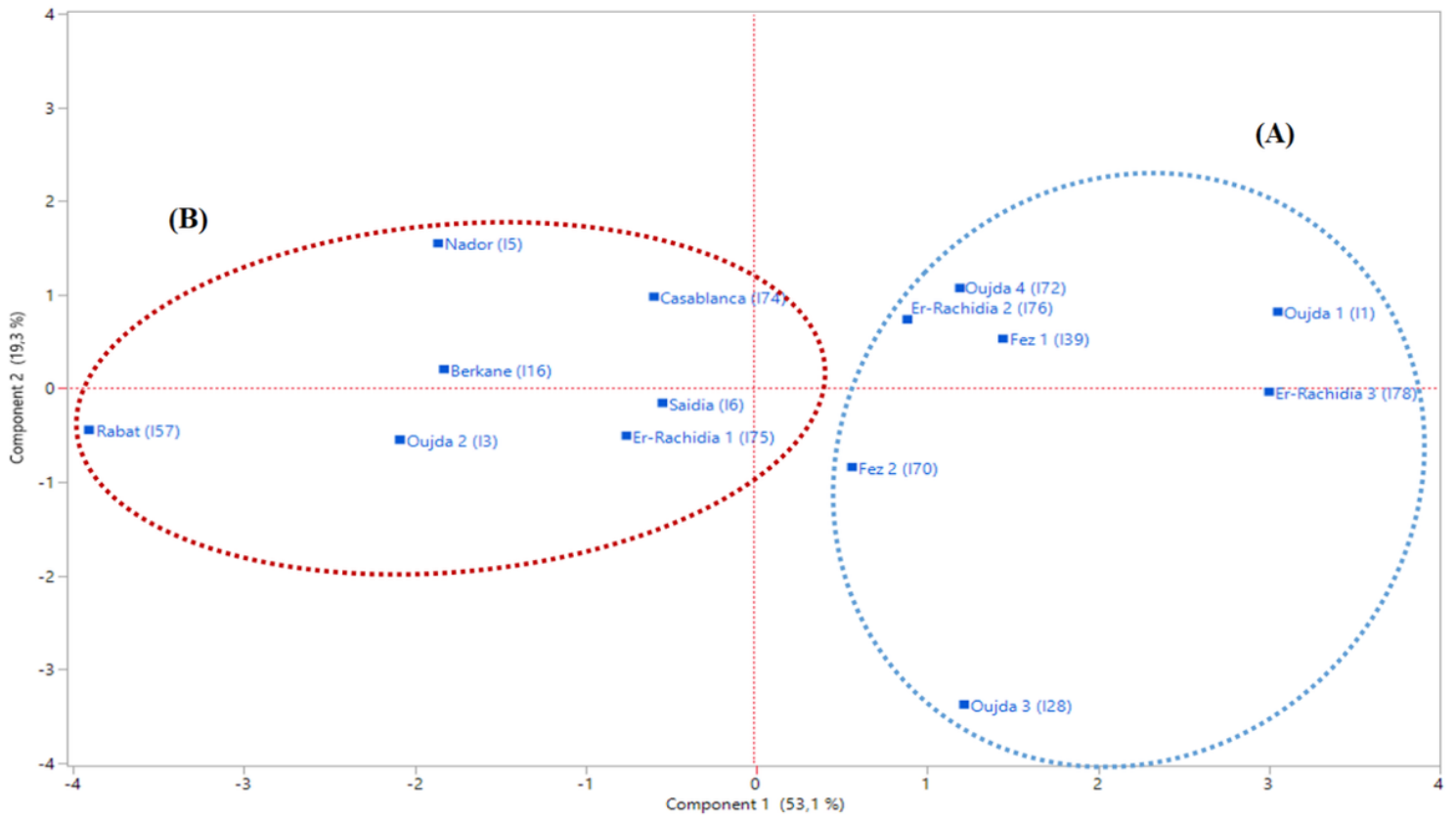


Figure 2

Distribution plot of different sampling sites in relation to phenotypic and symbiotic parameters according to the first two components.

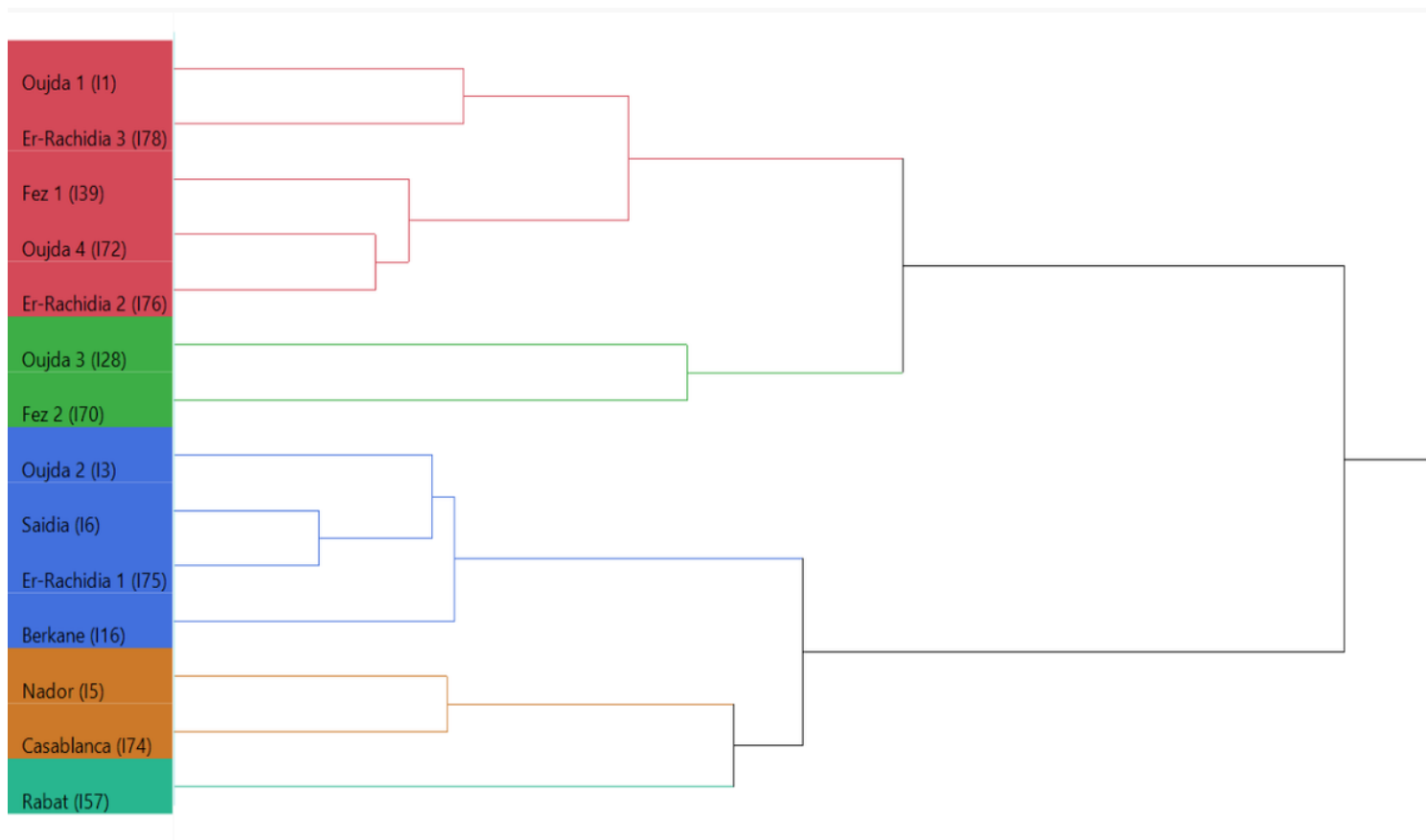


Figure 3

Classification plot obtained by hierarchical ascending classification (HAC) of the different sampling sites according to phenotypic and symbiotic parameters.

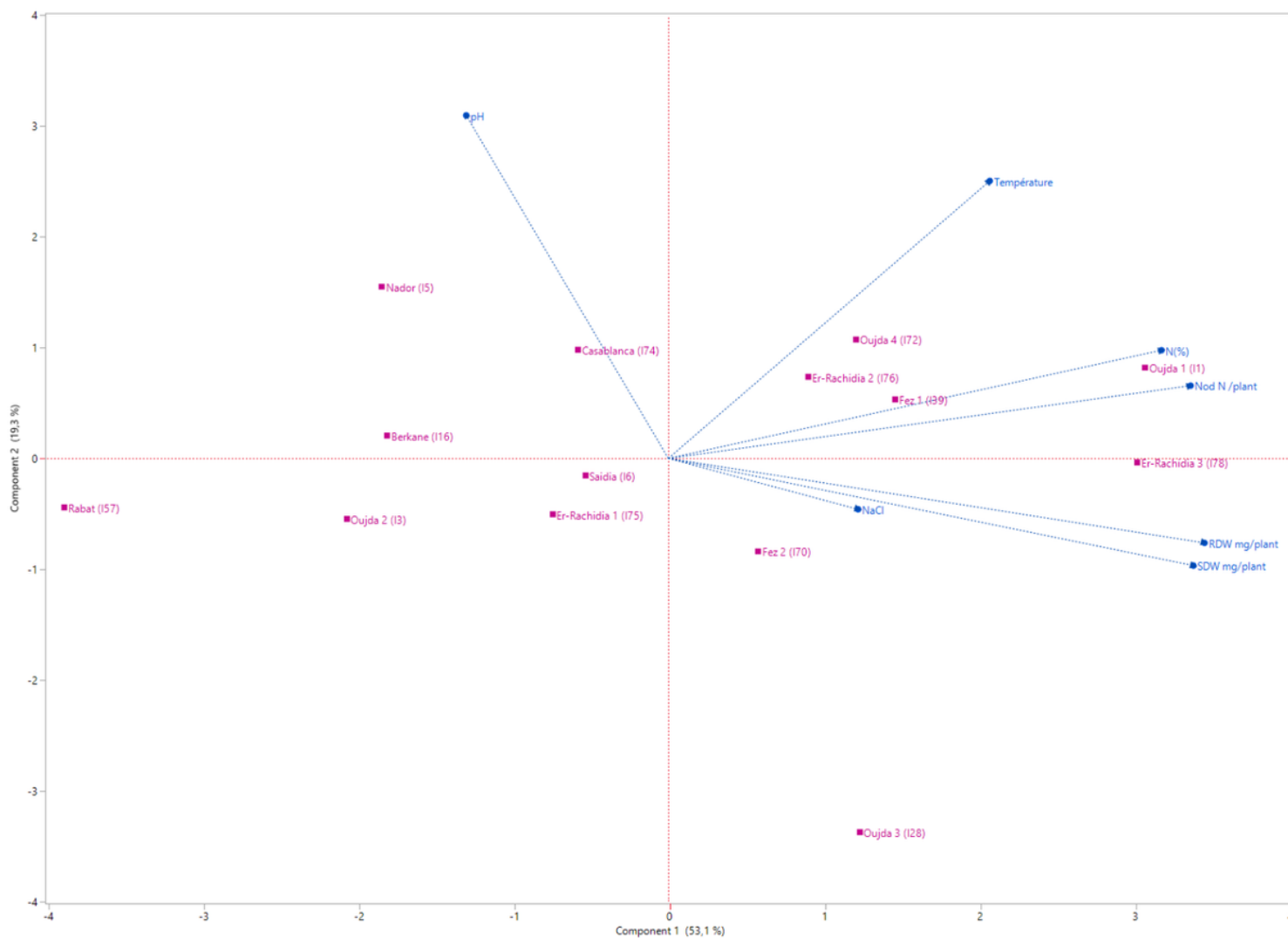


Figure 4

Biplot of the relationship between the distribution of different sampling sites and phenotypic and symbiotic parameters according to the first two components.